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(71) Applicant: **ORION-YHTYMÄ OY**  
**P.O. Box 65**  
**SF-02101 Espoo(FI)**

(72) Inventor: **Söderland, Hans Erik**  
**Salonkitie 19**  
**02940 Espoo(FI)**  
Inventor: **Syvänen, Ann-Christine Elizabeth**  
**Mannerheimintie 138 A 11**  
**00270 Helsinki(FI)**

(74) Representative: **Vossius & Partner**  
**Siebertstrasse 4 P.O. Box 86 07 67**  
**D-8000 München 86(DE)**

(54) **Method and reagent combination for determining nucleotide sequences.**

(57) The invention provides for detection of changes in a nucleotide sequence based on the determination of the nucleotide sequence over the region of interest. The first step of the process involves obtaining an amplified DNA sample in which at least one attachment moiety has been introduced into at least one strand of a target polynucleotide by a modified polymerase chain reaction. This can be accomplished by synthesizing DNA using the target nucleic acid polymer as a template and using at least one amplification primer, which includes an attachment moiety bonded to the oligonucleotide. The resulting amplification mixture is repeatedly cycled between a polymerization condition and a denaturation condition occurs for a number of cycles sufficient to produce copies of the target nucleic acid polymer in a quantity suitable for use in a DNA sequencing procedure to form an amplified sample comprising copies of the target nucleic acid sequence bonded to the attachment moiety. After amplification, the strands of target nucleic acid polymer carrying an attachment moiety are captured using a solid matrix coated with an attachment site to which the attachment moiety or a modification thereof can bind. In this way, the strands of target nucleic acid become

bound to the solid matrix and can be readily separated with the solid matrix from unbound reactants for sequencing.

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## METHOD AND REAGENT COMBINATION FOR DETERMINING NUCLEOTIDE SEQUENCES

The present invention relates to a method and a reagent combination for determining the nucleotide sequence over a defined polynucleotide region, and to the use of this method in identifying genetic variations.

The genetic information of living organisms is carried in the nucleotide sequence of their genome. In the process of gene expression the nucleotide sequence is translated to amino acid sequences, i.e. proteins. Minor changes in the nucleotide sequence, even a single base substitution, may result in an altered protein product. The altered quality or quantity of given proteins changes the phenotype (i.e. the observable characteristics) of the organism or the cell, which for instance may be observed as a development of a disease. It would therefore be significant if changes in nucleotide sequences in the genome of living organisms could be determined accurately and with such efficiency that large numbers of samples could be screened. This would afford opportunities for diagnosis of hereditary predispositions or disease, detection of somatic mutations in cancer, and selection of cells and strains for plant and animal breeding.

The detection of changes in the nucleotide sequence can be done in different ways. A method yielding very convincing results is the actual determination of the nucleotide sequence over the region of the genome containing the gene of interest. Compared to nucleic acid hybridization methods, the method of determination of the nucleotide sequence makes it unnecessary to know the actual mutation. It is only necessary to know the location of the region of interest and to compare this region in a sample with the same region in a "normal" standard.

Two basic approaches are in use for nucleotide sequence determination. These are the chemical method of Maxam-Gilbert and the chain termination method of Sanger. Both methods are well described in contemporary biochemistry text books. Common to these two methods is the requirement for submicrogram amounts of relatively pure target polynucleotide to be sequenced. In most cases this target polynucleotide is cloned by genetic engineering methods into suitable vectors for biological amplification in order to obtain sufficient quantities of nucleic acid polymer for analysis. The repeated replication reaction (see Kleppe et al., J. Mol. Biol. 56 (1971), 341-361) has provided another way of amplifying given polynucleotide regions *in vitro*. The method has later been applied by Mullis et al. (see EP 200,362). While this method provides substantial advantages in speed and efficiency over the cloning method of producing adequate quan-

ties of DNA, it does not provide a product which is directly suited for sequencing. Instead, the amplified region needs to be carefully purified e.g. by electrophoresis or ultrafiltration, from the amplification reaction mixture which if present during the sequencing process would be deleterious for the sequencing. These purification procedures tend to be time consuming arguing against their use in routine analysis.

Thus, the technical problem underlying the present invention is to provide a method which permits a fast reliable, simple and optionally automatic detection of changes in a nucleotide sequence which is well adapted for use as a routine diagnostic tool. This technical problem is solved by providing the method of the present invention and the kits to be used therein. These embodiments are characterized in the claims. When applying the method of the present invention, there is no necessity for cloning the target polynucleotide into specific vectors. Isolation of enzymatically amplified target polynucleotide is simple and there is no work consuming purification steps e.g. ultrafiltration or gel electrophoresis. Furthermore the sequencing reactions can be performed on immobilized templates, which provides advantages in process control and possibilities for automation and simultaneous sequencing of both strands of target polynucleotide.

The method of the invention for detection of changes in a nucleotide sequence is based on the determination of the nucleotide sequence over the region of interest. In accordance with the invention, the first step of this process involves obtaining an amplified DNA sample in which at least one attachment moiety has been introduced into at least one strand of specific target polynucleotide by a modified polymerase chain reaction. This can be accomplished by combining a sample containing the target nucleic acid polymer with a mixture of nucleotide monomers (dATP, dCTP, dGTP, and dTTP), a DNA polymerase, the first and second amplification primers. Each of the amplification primers comprises an oligonucleotide having a region that is complementary to the hybridizes with a different strand (i.e., one or the other of the two complementary strands) of the target nucleic acid polymer and that is effective as a primer for DNA polymerization by the DNA polymerase. At least one of the amplification primers further comprises a first attachment moiety bonded to the oligonucleotide. The resulting amplification mixture is repeatedly cycled between a first condition under which DNA polymerization occurs and a second condition under which denaturation of double

stranded DNA to single stranded DNA occurs for a number of cycles sufficient to produce copies of the target nucleic acid polymer in a quantity suitable for use in a DNA sequencing procedure to form an amplified sample comprising copies of the target nucleic acid sequence bonded to the attachment moiety. A third incubation condition under which annealing of single to double stranded DNA occurs, particularly annealing of the single stranded amplification primers to the single stranded template DNA, may advantageously be incorporated before each polymerization step.

After amplification, the strands of target nucleic acid polymer carrying an attachment moiety are captured using a solid matrix coated with an attachment site to which the attachment moiety or a modification thereof can bind. In this way, the strands of target nucleic acid become bound to the solid matrix and can be readily separated with the solid matrix from unbound reactants.

The purified copies of the target polynucleotide are denatured either before immobilization or following separation of the solid matrix from the unbound reactants, and the sequence of the amplified target polymer is determined. For example, chain termination sequencing reactions can be initiated on the denatured copies of the target polynucleotide, which are now acting as templates. The newly synthesized sequencing reaction products are then released from the template and separated and detected by size.

The present invention also encompasses a reagent combination or kit for practising the method of the invention. While the precise packaging will depend on the combinations of attachment moieties and attachment sites selected, such a kit will in general include an amplification primer having an attachment moiety included and a support adapted to immobilize the amplification primer.

The method of the present invention is useful for determining in a target nucleic acid at least one potential nucleotide variation, said variation being a nucleotide replacement, deletion or insertion.

Thus, the method of the present invention is applicable in forensic analysis or the analysis of hereditary predispositions or diseases. In some of these analyses the nucleotide variation is a somatic mutation.

From the above it is evident that the method of the present invention can also be applied in selecting cells, strains or species in plant or animal breeding or for selecting microorganisms displaying a desired genotype.

The kits of the present invention are suitable for performing the above mentioned methods.

Fig. 1 is a schematic drawing of the sequence of steps used in one embodiment of the invention; and

Fig. 2 is a schematic drawing of possible regions of complementarity between a target nucleic acid and a sequencing primer.

The present invention discloses a method to determine the nucleotide sequence of previously defined regions in complex nucleic acid mixtures. In a preferred embodiment, two known basic methods are utilized: the chain-termination sequencing procedure of Sanger and the repeated replication reaction (RRR). The invention also utilizes the affinity based hybrid collection procedure described in the parent application, U.S. Patent Application Serial No. 024,604, which is incorporated herein by reference. The novel combination of these methods in the present invention yields a facile and efficient process for sequencing nucleic acids which is well suited to the detection of genetic defects.

In accordance with the invention, a small sample of target nucleic acid is first amplified using a repeated replication reaction (RRR) in which one or both of the amplification primers are modified to include an attachment moiety, for example, one member of an affinity pair. The target sequence of interest is amplified with a suitable number of cycles of this modified RRR-reaction. Then the sample may be treated to render double-stranded nucleic acids single stranded, whereafter all molecules modified with a given attachment moiety are bound to a solid matrix coated with a complementary attachment site, e.g. the other component of an affinity pair. The matrix is then washed to remove all unbound material. It is also possible to attach amplified DNA to the matrix before denaturation. In that case, the denaturation of double-stranded adsorbed nucleic acid molecules to single stranded form would be done after immobilization (attachment). This approach also makes it possible to elute the essentially pure complementary strand even though it may not have been synthesized with an attachment moiety.

Independent of the stage at which the denaturation step is done, the result is a solid support onto which single-stranded nucleic acid is bound from one end. This solid support, preferably microbeads, is then introduced into the reaction mixtures of the chain termination sequencing process, one reaction mixture for each nucleotide, A, C, G, or T. The polymerization step is allowed to proceed on the beads. Then the beads may be washed if desired, whereafter the polymerization product is eluted from the beads and analyzed by gel electrophoresis as described for a number of manual, semimanual or automated sequencing protocols. In a modification of this process the essentially pure complementary strand eluted from matrix bound modified strand is used as the sequencing template. If both amplification primers are modified with a different attachment moiety and the resulting

amplified DNA is "attached" to different physically separable matrices, both strands can be introduced into the same sequencing reaction mixture and the matrices to which they are attached can be separated from each other as late as just prior to the final denaturation and gel electrophoresis. This possibility of sequencing both strands simultaneously with separation of the two not occurring unit just prior to electrophoresis decreases the amount of sequencing work by almost a factor of two. The different modes of practising the present invention are now detailed.

(a) Modification of target nucleic acid fragments with attachment moieties.

The source of the target nucleic acid (DNA or RNA) to be analyzed by the method of the invention can be any human cell, animal cell, plant cell or microbe. The target nucleic acid can be isolated from biological samples by conventional nucleic acid purification methods but according to the present invention it is also possible to use unpurified biological samples directly.

In the method of the invention attachment moieties are introduced into the target polynucleotide by the modified repeated replication reaction with the aid of two oligonucleotide primers per region to be amplified. At least one of these "amplification" primers has been modified with an attachment moiety. For purposes of this application and the claims hereof, an affinity moiety is a component having affinity for another component which forms an affinity pair by preferentially and selectively binding with that other component. For example, biotin/avidin or streptavidin, complementary polynucleotides, including homopolynucleotides such as poly dA/poly dT and poly dG/poly dC, hapten/antibody, and metal/chelate, are such affinity pairs. Any component pairs with strong affinity for each other can function as an affinity pair. An attachment moiety is an affinity moiety or a moiety providing a site for the attachment of an affinity moiety.

The oligonucleotide primers may be synthesized by standard chemical methods. Also recombinant DNA techniques may be used for the preparation of primers. The size of the primers is preferably between 14 and 40 bases, but primers of at least 8 bases or considerably longer than 40 bases may also be used. The primers are modified with the attachment moieties using chemical or enzymatic or any other methods. Preferably the attachment moiety is attached to the 5' end of the primer. Other sites of attachment are also possible, provided that the base pairing property of the primer and its primer function are retained.

In one embodiment of the method of the invention two modified primers are used. The primers must in this case be modified with different attachment moieties.

In the method of the invention the amplification primers, one or both modified, the target nucleic acid, mononucleoside triphosphates and a nucleic acid synthesizing enzyme, such as DNA polymerase, are combined. Repeated cycles of denaturation of the target, annealing of the primers to the target strands and enzymatic elongation of the primers are carried out according to any repeated replication reaction protocol for as many cycles as necessary to obtain a quantity of nucleic acid sufficient for use in the DNA sequencing reaction to be used. For use in chain termination sequencing reactions, at least  $10^{10}$  molecules of DNA are preferably obtained.

As a result of the process, copies of the original target polynucleotide, now modified with attachment moieties, are produced by incorporation of the modified primers into the newly synthesized polynucleotide molecules. When only one of the primers is modified, polynucleotide molecules with one strand modified with an attachment moiety are synthesized. The use of two differently modified primers, each adapted to hybridize with a different strand of the target nucleic acid, produces polynucleotide molecules with one strand modified with one attachment moiety and the other strand modified with a different attachment moiety. A special case is obtained when one of the primers is modified with a polynucleotide attachment moiety. In this case the other strand becomes modified with the complementary sequence during the RRR-process which can then serve as an attachment moiety for the second strand. The size and amount of the modified target DNA produced is limited only by the capacity of the repeated replication reaction.

(b) Isolation of single stranded target polynucleotide

After synthesis the modified target polynucleotide is captured on a solid matrix, to which an attachment site has been attached. For purposes of this application and the claims hereof, an attachment site is a component of an affinity pair which is selected so as to preferentially and selectively bond with the affinity moiety used as or subsequently bonded to the attachment moiety incorporated in the amplification primer. When one strand of the target polynucleotide is modified with an affinity moiety, one solid matrix is used, and when both strands are modified, two different solid matrices are used. Preferably the two matrices are selected such that they can be physically sepa-

rated from each other. Alternatively, the capturing of the two differently modified strands can be done sequentially on two different solid matrices.

The solid matrix can be of any format, such as beads, microparticles, the surface of a microtitration well or a test tube, a dipstick or a filter. The material of the matrix may for instance be polystyrene, cellulose, latex, nitrocellulose, nylon, polyacrylamide, dextran or agarose. The only prerequisite for the material of the matrix is that the attachment site can be attached to it. The binding capacity of the matrix should enable capture of a sufficient amount of the modified target polynucleotide as well as the excess of the modified primer present, which will also be captured. However, the use of a matrix which easily can be divided into portions is preferred, e.g. a large number of matrix particles of lesser binding capacity as opposed to a few matrix particles of very high capacity.

The modified target nucleic acid can be brought into contact with the affinity matrix directly in the reaction mixture in which the amplification was carried out. The mixture may also be diluted, or its composition may be changed to obtain conditions favorable for the reaction between the components of the affinity pair.

When two affinity pairs are used the target polynucleotide is preferably denatured before the affinity capture reaction to enable separate collection of the two strands on different affinity matrices. If these two different affinity matrices can be physically separated from each other e.g. after sequencing reaction stage, the two matrices can be handled as one in the capturing stage and in the sequencing reaction stage. When the target is modified in one strand only, it is optional to denature the target before the capturing reaction.

The attachment moieties of the target polynucleotide are allowed to react with the immobilized complementary attachment site for a sufficient time period to recover a substantial portion of the amplified target nucleic acid, which may vary depending on the kinetics of the binding reaction. In the case that the attachment moiety is not directly bondable to the attachment site this will further involve the introduction of a linking moiety, a molecule with separate binding sites for the attachment moiety and the attachment site which can form a link between the two. After the capturing reaction the bound target polynucleotide is separated from unbound material, including excess mononucleoside triphosphates, unmodified primer, enzyme, salts etc. by washing the matrix under suitable conditions. The separation procedure depends on the physical form of the matrix, and may for instance be centrifugation, magnetic separation, a column procedure or rinsing of the surface of the

matrix or combinations thereof.

After the washing procedure, the matrix is treated under conditions that render the bound target polynucleotide single stranded. Any conditions which denature double stranded polynucleotide, such as heat, alkali or enzymes, but do not affect the bonds between the components of the affinity pair, may be used. This step is compulsory if the target polynucleotide was not denatured before the capturing reaction. When denaturation was done before the capturing reaction this step is advantageous, but optional, to ensure that target molecules that may have reannealed during the capturing step are denatured. The exception, of course, is the case in which the attachment moiety and attachment site are complementary nucleic acid strands. In this case, denaturation must precede attachment since the link to the support would otherwise be lost.

Furthermore, in another variant of the invention, the denaturation step performed at this stage provides a new convenient method for the isolation of one strand of the target synthesized by the polymerase chain reaction. The isolated strand is free of excess mononucleoside triphosphates, primers and other components of the RRR-reaction and can be sequenced by conventional sequencing in solution or used for some other purpose.

### (c) The sequencing reaction

According to the present invention the sequence determination of the isolated amplified polynucleotide fragments may be performed using the chain termination method. The method is based on the use of deoxynucleotide analogues which are randomly incorporated by a polynucleotide synthesizing enzyme into a growing polynucleotide strand to give specific chain termination. Chain termination can also be achieved using very low concentrations of one of the deoxynucleoside triphosphates.

The necessary components of the chain termination sequencing reaction are a sequencing primer, a polynucleotide synthesizing enzyme, the chain terminating dideoxynucleotides, deoxynucleotides and a detectable label. According to the present invention the different standard laboratory protocols for sequencing are applied to sequencing the immobilized target polynucleotide. The order of addition of the components of the sequencing reaction may vary, depending on the affinity matrix used and on the chosen protocol.

As shown in Fig. 2, the sequencing primer may be distinct from or equal to the primer used in the RRR amplification reaction. If it is distinct from the amplification primer it may be completely located

between the region of interest in the target nucleic acid polymer and the 3' end of the amplification primer (A), or it may be partially overlapping the amplification primer in such a way that its 3' end anneals at least one nucleotide, but preferably more than 3 nucleotides of the 5' end of the target nucleic acid polymer (B). The sequencing primer may also fall entirely within the amplification primer (C) or be the same as the amplification primer (D).

The choice of sequencing primer depends on the purpose of the analysis. A primer distinct from the RRR primer will increase the specificity of the sequencing reaction, because sequencing of wrong fragments possibly produced in the RRR is avoided. Thus, a sequencing primer distinct from the amplification primer is preferred. On the other hand, more prior information of the sequence of the amplified fragment is needed to make such a primer.

The label for detection of the fragments produced in the sequencing reaction can be introduced by using a labelled sequencing primer. The label may be situated at the 5'-end or internally. It may be an isotope, such as  $^{32}\text{P}$ , or a fluorescent group, or any other detectable label, or a group providing a site for later attachment of a detectable label. The label can also be introduced using deoxynucleotide derivatives labelled with an isotope, such as  $^{35}\text{S}$  or  $^{32}\text{P}$ , or with fluorescent or other detectable labels. The labeling moiety is incorporated in an amount such that it is incorporated in substantially all of the nucleic acid synthesized, even very short fragments.

The matrix carrying the polynucleotide fragment is suspended into a suitable solution containing the sequencing primer. The primer is preferably added in equal or excess molar concentration to the target polynucleotide. The primer is allowed to anneal to the target polynucleotide at suitable temperature and for a sufficient time depending on the size and amount of the primer used. It should be noticed that when both strands of target polynucleotide are modified and bound to different affinity matrices, which can be physically separated from each other and handled now as one, two different sequencing primers are used.

If a dividable affinity matrix, such as beads, is used it is convenient to divide the sample after annealing of the primer into four parts of the individual sequencing reactions. If an undividable affinity matrix, such as a microtitration well is used, four parallel reactions are prepared at the affinity-capturing step. It is also possible to carry out the four reactions sequentially on the same carrier by eluting the product of each reaction before carrying out the next one. In certain cases enough information is obtained using only one, two, or three reactions. For example, if a genetic defect is known to

arise from a point mutation at a single specific site which is normally C, it may be sufficient to run only the C reactions since the actual identity of a non-C nucleic acid residue at this point is not needed. The relevant fact is that the residue is non-C.

After annealing, a polynucleotide synthesizing enzyme, such as *E. coli* DNA polymerase I or T7 DNA polymerase, and mixtures containing the chain terminating dideoxynucleotides, deoxynucleotides, and if unlabelled primers were used, labelled deoxynucleotides at suitable ratios which depend on the enzyme used and on the size of the target, are added. The polymerization reaction is allowed to proceed for a sufficient time period to achieve a substantially complete distribution of fragment sizes at a temperature that depends on the enzyme and conditions used. Finally, a chase reaction with deoxynucleotides is carried out to complete the synthesis of the polynucleotide strands which have not been terminated. The reaction is then stopped.

In the method of the invention the affinity matrix may be washed at this stage to remove the components of the sequencing reaction. The composition of the mixture may also thus be changed to obtain the most favorable conditions for the analysis of the synthesized fragments. If both strands were modified and bound to two different affinity matrices, the matrices are physically separated at this stage, e.g., by a magnetic field. The target DNA is rendered single stranded and the synthesized fragments from the four sequencing reactions are separated by electrophoresis or another suitable method. In the method of the invention the affinity matrix can be removed before electrophoresis, or when the affinity matrix is a microparticle it can be loaded together with the sample into the slots of the gel for the electrophoresis step. This gives an advantage in sample handling. In the electrophoresis step the size distribution of the fragments is analyzed. The detection procedure may vary depending on the label used. With isotopes the gel is prepared by fixing the drying and exposed to an x-ray film according to standard procedures. With fluorescent labels the fragments are detected using automatic equipment developed for this purpose. The size distribution of the polynucleotide fragments produced by the sequencing reaction produces a pattern called a "sequencing ladder" which gives the nucleotide sequence of the target polynucleotide.

Reagents for use in practising the method of the invention may be packaged in kit form. For example, kits might be prepared comprising packaged combinations of one or two amplification primers which include attachment moieties and the corresponding solid supports. Kits might also be prepared which further include a sequencing prim-

er or a reference means for comparison of the determined sequence of the nucleic acid polymer with the sequence of a standard. Examples of reference means include package inserts showing the sequencing ladder obtained from sequencing of the standard, or a sample of the standard itself. If the standard is supplied, it advantageously has an attachment moiety attached to it so that it can be handled in a similar manner to the target for sequencing.

The arrangement of the reagents within containers of the kit will depend on the specific reagents involved. Of course, each reagent can be packaged in an individual container, but various combinations may also be possible.

For example, if the attachment moiety and the attachment site used will not bind to each other under the conditions of the RRR-reaction, both can be provided in a single container. This might be the case, for example, if a linking moiety was used.

The present invention is illustrated more specifically with the following examples, which are not intended to limit the scope of the invention.

### Example 1

Determination of the nucleotide sequence of a biotinylated cytomegalovirus DNA fragment immobilized on avidin-microparticles

### Synthesis of the Primers

The primers were synthesized on an Applied Biosystems 381A DNA synthesizer by the methoxy phosphoramidite method. The primers (denoted 25A and 25B) were 25-mers with the following nucleotide sequence:

25A: 5'-TCG CAA GCT CTT TCC CGG CCT GGC T

25B: 5'-GCT CTG CGC GAA CAT GTA GTC GGC C

A 5'-terminal aminogroup was added to the 25A-primer as the last step in the synthesis using the aminolink II reagent (Applied Biosystems, P/N 400808). The aminogroup was biotinylated with sulfo-NHS-biotin (Pierce Chemical Co.). The biotinylated oligonucleotide was purified by HPLC on a reversed phase C-18 column.

### The Target DNA

The target DNA was a fragment of the long unique region of the cytomegalovirus (CMV) genome at map position 0.30-0.35, i.e. the 12.1 kb Hind III-fragment of AD 169 CMV DNA(ATC VR-538) cloned in the pAT153 vector. The target plasmid was linearized with the restriction enzyme Eco RI. The primers flank a 115 bp region in the CMV Hind III fragment.

### Primer Extension Reaction-Amplification

Biotin was introduced into a 165 base pair fragment of the CMV DNA by 25 cycles of amplification by the repeated replication reaction (RRR) using the 5'-biotinylated primer 25A and the unmodified primer 25B. The amplification was carried out with  $3 \times 10^5$  molecules of the linearized CMV Hind III fragment, 100 pmol each of the primers, 200  $\mu$ M each of the four mononucleoside triphosphates (dATP, dCTP, dGTP, dTTP) in 100  $\mu$ l of a solution of 10 mM Tris-HCl, pH 8.35, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.1 mg/ml gelatin in 0.5 ml Eppendorf tubes under a layer of viscous paraffin. The target DNA was boiled for 5 min. in the reaction mixture to render it single stranded, after which 2U of *Thermus aquaticus* DNA-polymerase (New England Biolabs) was added. The tubes were then processed for 25 cycles as follows: Primer annealing at 55°C for 1 min., extension of the primers at 72°C for 3 min., denaturation of the template at 95°C for 1 min 15 sec.

### Capture of the Biotinylated Amplified DNA on Avidin-coated Polystyrene Particles.

A 25  $\mu$ l sample, which contained about  $10^{10}$  molecules, i.e. 0.02 pmoles, of the amplified DNA-fragment, which was double stranded and contained a 5'-biotin in one strand, was collected from the reaction mixture by incubation with 5  $\mu$ l of a 5% solution of avidin-coated polystyrene particles (0.8  $\mu$ m, Pandex Laboratories) for 1 hour at room temperature. The beads were collected by centrifugation in an Eppendorf centrifuge for 3 min. and washed three times by vortexing with 1 ml of phosphate buffered saline (PBS), pH 7.5, containing 0.1% triton-X-100, followed by centrifugation as above. The bound DNA was denatured by treatment with 200  $\mu$ l of 0.15 M NaOH for 15 min. at 37°C, the particles were collected and washed twice with 1 ml of 0.1% triton-X-100, and finally once with 1 ml of 0.01% triton-X-100.

### Sequencing of the Immobilized DNA Fragment

The microparticles carrying the DNA fragment were suspended into 10  $\mu$ l of 40 mM Tris-HCl, pH 7.5, 20 mM MgCl<sub>2</sub>, 50 mM NaCl, 100  $\mu$ g/ml bovine serum albumin, containing 0.06 -0.08 pmoles of the 25B primer. The primer was allowed to anneal for 10 min. at 55°C followed by 30 min. at 37°C. Then 2  $\mu$ l of <sup>35</sup>S-labelled dCTP (600 Ci/mmol; Amersham) and 1  $\mu$ l (5 units) of *E. coli* DNA polymerase (the Klenow fragment; Boehringer Mannheim) were added. The suspension was divided into four test tubes, 2.5  $\mu$ l in each, for the individual sequencing reactions. 2  $\mu$ l of deoxy- and dideoxynucleotide mixtures were added to yield the following final concentrations:

A-reaction: 125  $\mu$ M dGTP, dTTP, 16  $\mu$ M dATP, 250  $\mu$ M ddATP

C-reaction: 125  $\mu$ M dGTP, dTTP, dATP, 20  $\mu$ M ddCTP

G-reaction: 125  $\mu$ M dTTP, dATP 16  $\mu$ M dGTP, 200  $\mu$ M ddGTP

T-reaction: 125  $\mu$ M dGTP, dATP 16  $\mu$ M dTTP, 500  $\mu$ M ddTTP.

The reaction was allowed to proceed for 20 min. at room temperature. Then 2  $\mu$ l of a 2 mM solution of the four dNTPs were added, and the chase reaction was allowed to proceed for 20 min. at room temperature. The reaction was stopped by adding 4  $\mu$ l of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol. The samples were heated for 3 min. at 98°C. The tubes were centrifuged and 3  $\mu$ l of the supernatant was loaded on an 8% polyacrylamide sequencing gel. The samples were electrophoresed, the gel removed, fixed and exposed to an x-ray film according to standard procedures. After development clear sequencing ladders were observed.

### Example 2

The whole procedure was carried out identically as in Example 1, with the exception that the samples from the sequencing reaction were loaded directly onto the gel without prior removal of the microparticles. Sequencing ladders identical to those in Example 1 were obtained.

### Example 3

In this example <sup>32</sup>P-labelled primers were used in the sequencing reactions. The procedure was carried out as in Example 1, with the following

exceptions:

The sequencing primer (25B) was labelled using gamma-<sup>32</sup>P-ATP and polynucleotide kinase according to a standard procedure. 2 pmoles of oligonucleotide primer and 14 pmoles of gamma-<sup>32</sup>P-ATP (> 5000 Ci/mmol; Amersham) were used per labelling reaction. Primers with specific activities of 10<sup>7</sup> cpm/pmol were obtained.

For the sequencing reaction the microparticles carrying about 0.02 pmoles of the amplified DNA fragment were suspended in the sequencing buffer (see Example 1) containing 0.1 pmole of the labelled 25B-primer. The primer annealing reaction was as in Example 1. Then 1  $\mu$ l (5 units) of DNA polymerase was added and the suspension was divided into four parts, 2.5  $\mu$ l each. 2  $\mu$ l of deoxy- and dideoxy-nucleotide mixtures were added to yield the following final concentrations:

A-reaction: 125  $\mu$ M dCTP, dGTP, dTTP, 16  $\mu$ M dATP and 250  $\mu$ M ddATP

C-reaction: 125  $\mu$ M dATP, dGTP, dTTP, 16  $\mu$ M dCTP and 80  $\mu$ M ddCTP

G-reaction: 125  $\mu$ M dATP, dCTP, dTTP, 16  $\mu$ M dGTP and 200  $\mu$ M ddGTP

T-reaction: 125  $\mu$ M dATP, dCTP, dGTP, 16  $\mu$ M dTTP, 500  $\mu$ M ddTTP.

The chain termination and chase reactions, as well as analysis of the produced fragments were carried out as in Example 1. Clear sequencing ladders were obtained also in this example.

### Example 4

In this example the RRR-primer (amplification primer) and sequencing primer used were non-identical. The sequencing primer was located at a 78 base distance from the amplification primer toward the biotinylated 5'-end of the amplified fragment.

The PCR-amplification was carried out with the biotinylated primer 25A and a 25-mer primer, denoted 25C, with the following sequence:

5'-CAG GGT CAC TGA CAA CAG CCG CCC T.

The primers 25A and 25C flank a 209 bp sequence in the CMV-genome.

The amplification and capturing procedures were as described in Example 1. The primer 25B was labelled with <sup>32</sup>P and used as sequencing primer at the conditions described in Example 3. The obtained sequence was identical to that one obtained in Example 3.

### Example 5

All processes were carried out as in Example 1



except for the affinity-capturing step. In this example magnetic beads were used as the affinity matrix. The beads were 2  $\mu$ m in diameter and had a paramagnetic nucleus with a spherical polystyrene shell coated with streptavidin. The beads (Dynabead 280-Streptavidin) were obtained from Dynal, Norway. Before use the beads were washed once with phosphate buffered saline (PBS).

As in Example 1, 25  $\mu$ l of the sample containing the amplified DNA fragment was analyzed. 5  $\mu$ l of a 4% suspension of the magnetic beads in PBS were added and the capturing reaction was allowed to proceed for 30 min. at 37° C in Eppendorf tubes in a rotating mixer. The beads were separated from the solution for 1 min. using a standard laboratory magnet and the solution was discarded. The beads were washed three times with 1 ml of PBS by vortexing followed by magnetic separation as above. The bound DNA was denatured by treatment with 200  $\mu$ l of 0.15 M NaOH for 15 min. at 37° C. The beads were collected and washed twice with PBS and once with 40 mM Tris-HCl, pH 7.5, 20 mM MgCl<sub>2</sub>, 50 mM NaCl. The sequencing of the immobilized DNA fragment was as in Example 1.

#### Example 6

The RRR-amplification and capturing procedures were carried out as in Example 1. In this example the second strand of the amplified DNA fragment obtained at the denaturation step was sequenced.

The 200  $\mu$ l 0.15 M NaOH supernatant containing the DNA-strand eluted from the affinity matrix was neutralized by the addition of 20  $\mu$ l of 1.5 M acetic acid and 12  $\mu$ l of 3 M sodium acetate (pH 7). The DNA was precipitated with ethanol and dissolved in 10  $\mu$ l of the sequencing buffer (see Example 1) containing the sequencing primer. The sequencing procedure was carried out at the conditions specified in Example 1. Clear sequencing ladders were again obtained.

#### Example 7

Identification of genetic polymorphism of the apolipoprotein E by sequencing immobilized biotinylated apolipoprotein E DNA fragments

This example is given to illustrate the convenience of the method of the claimed invention for the diagnosis of genetic diseases. The method was applied to the analysis of the genetic variation of

the apolipoprotein E (apo E). This protein plays an important role in the lipoprotein metabolism. Three common isoforms of apo E (E2, E3 and E4), encoded by three different alleles ( $\epsilon$ 2,  $\epsilon$ 3 and  $\epsilon$ 4), exist in the population. The genetic variation of these alleles is due to single base substitutions at amino acid positions 112 and 158. This genetic polymorphism of apo E may explain as much as 10 % of the individual variation of serum cholesterol levels and is thus of major clinical significance.

#### Synthesis of the primers

Four primers (P1 -P4) were synthesized on an Applied Biosystems 381A DNA synthesizer. The nucleotide sequence of the primers and their location (given as nucleotide numbers) on the apolipoprotein E gene (Apo E) were:

P1: 5'-AAG GAG TTG AAG GCC TAC AAA T (3615 - 3637)

P2: 5'-TCG CGG GCC CCG GCC TGG TAC A (3893 - 3914)

P3: 5'-GAA CAA CTG AGC CCG GTG GCG G (3649 - 3671)

P4: 5'-GGA TGG CGC TGA GGC CGC GCT C (4012 - 4034)

A 5'-aminogroup was added to the primer P2 with the aminolink II reagent (Applied Biosystems). The amino group was biotinylated using sulfo-NHS-biotin (Pierce Chemical Co.) and purified by reversed phase HPLC. The sequencing primer (P3) was labelled with [ $\gamma$ -<sup>32</sup>P]dATP and T4 polynucleotide kinase to a specific activity of a 4 x 10<sup>6</sup> cpm/pm.

#### The DNA Samples

Venous blood samples were obtained from patients of known Apo E phenotype attending the Lipid Outpatient Clinic of the University Central Hospital of Helsinki. Leukocytic DNA was extracted according to standard procedures.

#### Amplification

The DNA (100 ng per sample) was amplified with the P1 and P4 primers (final concentration 1  $\mu$ M) in 100  $\mu$ l of a solution of 0.2 mM each of dATP, dCTP, dGTP, dTTP, 20 mM Tris-HCl, pH 8.8, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, 0.1 % tween 20, 0.1 mg/ml gelatin and 2.5 units of *Thermus aquaticus* DNA-polymerase (Promega) in a

DNA thermal cycler (Perkin-Elmer /Cetus) for 25 cycles of 1 min. at 96°C and 2 min. at 65°C. A small aliquot (3 µl of a 1:100 dilution) of this first amplification mixture was transferred to a second amplification. This second amplification was carried out at the conditions described above and directed by a pair of nested primers one of which was biotinylated (biotinylated P2 and P3).

#### Affinity-capture of the Biotinylated Amplified Apo E DNA on Avidin-coated Polystyrene Particles

A 25 µl aliquot of the second amplification mixture was diluted to 50 µl with 0.15 M NaCl, 20 mM Na-phosphate buffer, pH 7.5 (PBS), after which 5 µl of a 5 % (w/v) suspension of avidin-coated polystyrene particles (0.8 µm, Baxter Healthcare Corp.) were added. The samples were kept at 20°C for 1 hour. The particles were collected by centrifugation for 2 min. in an Eppendorf centrifuge and were washed twice by vortexing with 1 ml of 15 mM NaCl, 1.5 mM Na-citrate, and twice with 1 ml of 0.1 % Tween 20 in PBS. The particles were treated twice with 0.15 M NaOH for 15 min. at 37°C, followed by two washes with 0.1 % Tween 20 in 50 mM NaCl, 40 mM Tris-HCl, pH 7.5 and a final wash with 0.01 % Tween 20 in 50 mM NaCl, 40 mM Tris-HCl, pH 7.5.

#### Nucleotide Sequencing of the Immobilized DNA Fragment

The particles carrying the DNA template were suspended in 10 µl of 50 mM NaCl, 20 mM MgCl<sub>2</sub>, 40 mM Tris-HCl, pH 7.5, containing 0.5 - 1 pmol of the sequencing primer P2 or P3. The tubes were heated at 65°C for 2 min. and allowed to cool slowly to room temperature. One µl of 0.1 M dithiothreitol, 2.5 µl of H<sub>2</sub>O and 2 µl (3.25 units) of T7 DNA-polymerase (Sequenase™, United States Biochemical Corp.) were added and the tubes were kept at 22°C for 3 min. An aliquot (3.5 µl) of the reaction mixture was transferred to four tubes containing 2.5 µl of chain termination mixture (80 µM dNTP, 8 µM ddNTP for the A and T reactions; 320 µM dNTP, 8 µM ddNTP for the C and G reactions). The tubes were incubated at 42°C for 6 min. and the reaction was stopped by adding 4 µl of a solution containing 95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue and 0.05 % xylene cyanol. The samples were heated at 80°C for 2 min. and the particles were spun down for 2 min. The products of the chain termination reactions were run on 6 % sequencing gels. The electrophoresis was carried out and the gel was pro-

cessed according to standard procedures. The correct alleles were identified from the sequencing ladders obtained. In heterozygous samples both alleles were identified as superimposed sequencing ladders at the position of the nucleotide variation.

#### Claims

1. A method for determining the sequence of a target nucleic acid polymer comprising:

(a) combining a sample containing the target nucleic acid polymer with a mixture of nucleoside triphosphates, a nucleic acid polymerase, and first and second amplification primers, each amplification primer comprising an oligonucleotide having a region that is complementary to the hybridizes with a different strand of the target nucleic acid polymer and being effective as a primer for nucleic acid polymerization by the nucleic acid polymerase to form an amplification mixture, wherein said first amplification primer further comprises a first attachment moiety bonded to the oligonucleotide;

(b) repeatedly cycling the amplification mixture between a first condition under which DNA polymerization occurs and a second condition under which denaturation of double stranded DNA to single stranded DNA occurs for a number of cycles sufficient to produce copies of the target nucleic acid polymer in a quantity suitable for use in a DNA sequencing procedure to form an amplified sample comprising copies of the target nucleic acid sequence bonded to the attachment moiety;

(c) adding to the amplified sample a plurality of first supports, each comprising a solid matrix and a first attachment site which is capable of immobilizing one strand of the target nucleic acid polymer through the first attachment moiety;

(d) allowing the first attachment sites to interact with the first attachment moieties such that the copies of the target nucleic acid in the amplified sample are immobilized on the first supports;

(e) separating the immobilized nucleic acids from the amplification mixture;

(f) denaturing the amplified nucleic acid sample to form single stranded nucleic acids; and

(g) determining the sequence of the single stranded nucleic acids.

2. A method according to claim 1, wherein the step of denaturing the amplified sample is performed prior to immobilizing the copies of the target nucleic acid on the support.

3. A method according to claim 1 or 2, wherein the sequence of the single stranded immobilized nucleic acids is determined using a chain termination method.

4. A method according to any one of claims 1 to 3, wherein said first attachment moiety and said

first attachment site are components of an affinity pair.

5. A method according to claim 4, wherein the affinity pair is selected from the group consisting of biotin/avidin, biotin/streptavidin, poly-dA/poly-dT, poly-dG/poly-dC, hapten/antibody and metal/chelate.

6. A method according to any one of claims 1 to 5, wherein said second amplification primer comprises a second attachment moiety different from said first attachment moiety.

7. A method according to claim 6, further comprising the step of adding to the amplified sample a plurality of second supports comprising a support matrix and a second attachment site capable of immobilizing one strand of the target nucleic acid polymer through the second attachment moiety.

8. A method according to claim 6 or 7, wherein said second attachment moiety and said second attachment site are components of an affinity pair.

9. A method according to claim 8, wherein the affinity pair is selected from the group consisting of biotin/avidin, biotin/streptavidin, poly-dA/poly-dT, poly-dG/poly-dC, hapten/antibody and metal/chelate.

10. A method according to any one of claims 6 to 9, wherein said first and second supports are concurrently present in the amplified sample and are selected so as to be physically separable from each other.

11. A method according to claim 10, wherein one of said first and second supports are magnetic.

12. A method according to claim 10, wherein one or both of said first and second supports is a dipstick.

13. A method according to any one of claims 1 to 12, wherein the sequence-determination process includes the steps of combining the single-stranded immobilized nucleic acids with a sequencing primer and a sequencing mixture comprising nucleoside triphosphates, one chain terminating nucleotide, a polynucleotide synthesizing enzyme and a labeling moiety which is incorporated during nucleic acid synthesis; allowing polymerization to occur for a period of time sufficient for enough chain termination events to occur to permit determination of the sequence to form a partially polymerized sample; adding a completion reagent comprising a mixture of nucleoside triphosphates to the partially polymerized sample; allowing polymerization to occur in the completion reagent for a period of time sufficient to substantially complete DNA synthesis; separating the completely polymerized sample from the completion reagent; releasing the polymerized nucleic acids from the support;

denaturing the polymerized nucleic acids to render them single stranded;

analyzing the size of the single stranded nucleic acid polymers to determine the sequence of the target nucleic acid polymer.

14. A method according to claim 13, wherein the sequencing primer is an oligonucleotide selected from among oligonucleotides complementary with at least a portion of the amplification primer and oligonucleotides complementary with a portion of the target nucleic acid polymer which is located between the 3' end of the amplification primer and a region of interest in the target nucleic acid polymer.

15. A method according to any one of claims 1 to 14, further comprising the step of incubating the amplification mixture at a temperature selected to promote annealing of nucleic acids before each polymerization step.

16. A kit for use in determining the sequence of a target nucleic acid polymer comprising in packaged combination

(a) a first amplification primer comprising an oligonucleotide which is complementary to and hybridizes with a portion of the target nucleic acid polymer and which is effective as a primer for enzymatic nucleic acid polymerization and a first attachment moiety; and

(b) a plurality of first supports, each comprising a solid matrix and at least one attachment site which is capable of immobilizing the oligonucleotide of the amplification probe through the first attachment moiety.

17. A kit according to claim 16, wherein the attachment moiety and the attachment site are components of an affinity pair.

18. A kit according to claim 17, wherein the affinity pair is selected from the group consisting of biotin/avidin, biotin/streptavidin, poly-dA/poly-dT, poly-dG/poly-dC, hapten/antibody and metal/chelate.

19. A kit according to any one of claims 16 to 18, wherein the attachment moiety does not form a bond directly with the attachment site, further comprising a linking moiety selected so as to form a bond between the attachment moiety and the attachment site.

20. A kit according to any one of claims 16 to 19, further comprising a sequencing primer wherein the sequencing primer is an oligonucleotide selected from among oligonucleotides complementary with at least a portion of the amplification primer and oligonucleotides complementary with a portion of the target nucleic acid polymer which is located between the 3' end of the amplification primer and a region of interest in the target nucleic acid polymer.

21. A kit according to claim 20, wherein the

sequencing primer further comprises a detectable label.

22. A kit according to any one of claims 16 to 21, further comprising a reference means for comparison of the determined sequence of the target nucleic acid polymer with the sequence of a standard.

23. A kit according to claim 22, wherein the reference means is a package insert showing the sequencing ladder obtained from sequencing of the standard.

24. A kit according to claim 22, wherein the reference means is a sample of the standard.

25. A kit according to any one of claims 16 to 19 or 22 to 24, wherein the oligonucleotide of the amplification primer is complementary to and hybridizes with a portion of the target nucleic acid polymer which is derived from a target organism.

26. A kit according to any one of claims 16 to 25, further comprising a second amplification primer comprising an oligonucleotide which is complementary to and hybridizes with a portion of the second strand of the target nucleic acid polymer and which is effective as a primer for enzymatic nucleic acid polymerization and a second attachment moiety different from the first attachment moiety; and a plurality of second supports each comprising a solid matrix and at least one attachment site which is capable of immobilizing the oligonucleotide of the amplification probe through the second attachment moiety.

27. A kit according to claim 26, wherein the attachment moiety and the attachment site are components of an affinity pair.

28. A kit according to claim 26 or 27 wherein the affinity pair is selected from the group consisting of biotin/avidin, biotin/streptavidin, poly-dA/poly-dT, poly-dG/poly-dC, hapten/antibody and metal/chelate.

29. A kit according to any one of claims 26 to 28, further comprising a reference means for comparison of the determined sequence of the target nucleic acid polymer with the sequence of a standard.

30. The method according to anyone of claims 1 to 15 for determining in the target nucleic acid polymer at least one potential nucleotide variation.

31. The method according to claim 30 for forensic analysis or for the analysis of hereditary predispositions or diseases.

32. The method according to claim 30 or 31 wherein the nucleotide variation is a somatic mutation.

33. The method according to claim 30 or 31 for the selection of cells and strains or species for plant an animal breeding or for the selection of microorganisms displaying a desired genotype.

34. The kit according to anyone of claims 16 to

29 for use in a method according to anyone of claims 30 to 33.

Fig. 1.

The sequence of steps used in one embodiment of the invention

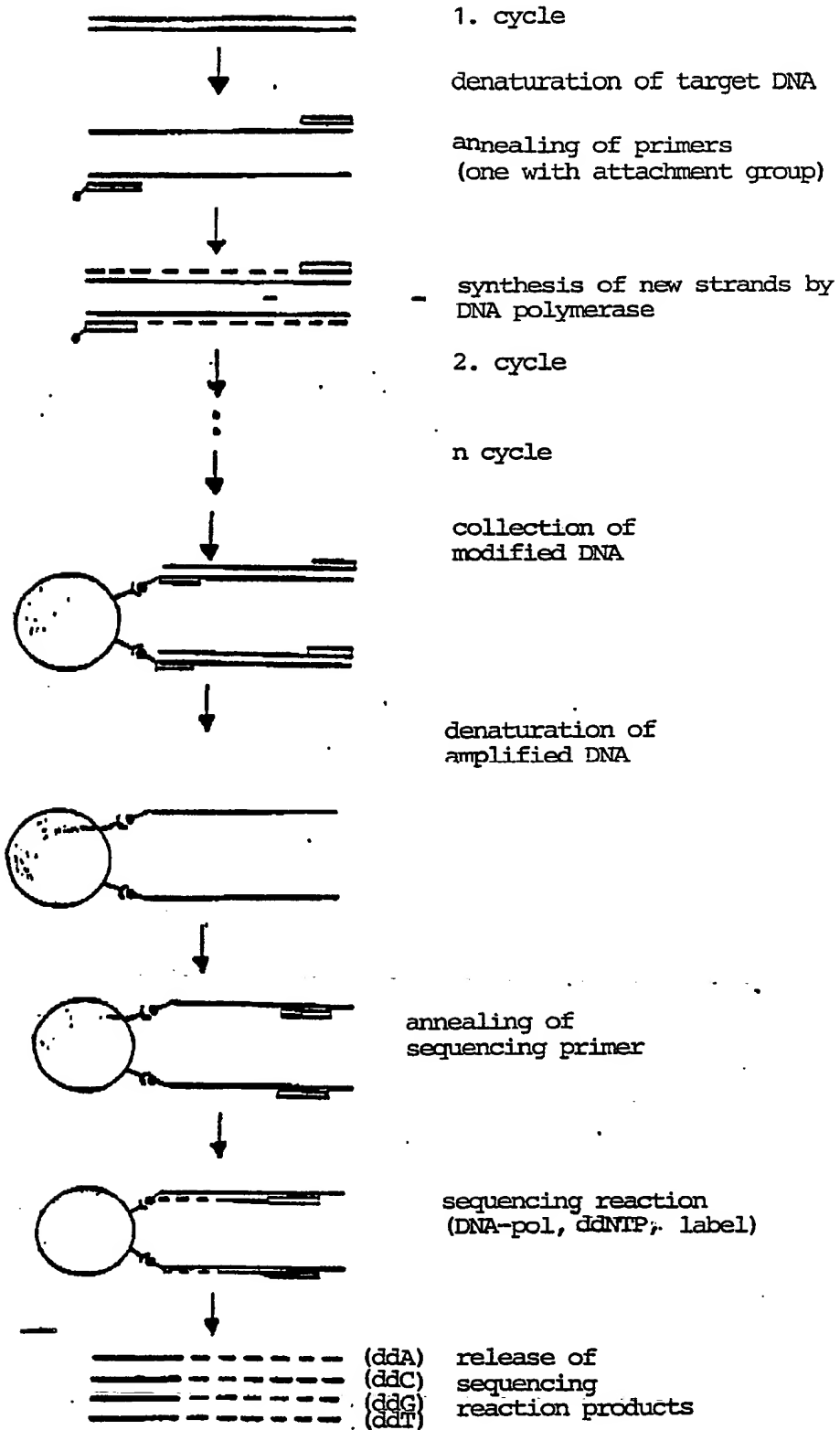


Fig 2.



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